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Binding of Ethidium Bromide to Double-Stranded Ribonucleic Acid†

R. J. Douthart,* J. P. Burnett, F. W. Beasley, and B. H. Frank

ABSTRACT: The interaction of ethidium bromide with double-stranded RNA (*Penicillium chrysogenum*) has been investigated using spectroscopic, spectropolarimetric, hydrodynamic, and thermal melting techniques. The binding isotherms (Scatchard plots) are dependent on ionic strength. The apparent binding constants and number of binding sites are quite similar to those found for DNA under similar conditions (Waring, M. J. (1965a), *J. Mol. Biol. 13*, 269). Hydrodynamic studies of the dye–RNA complex show a 53% increase in its viscosity increment, a 13% decrease in its relative sedimentation coefficient, and a decrease in its buoyant density in Cs_2SO_4 as compared to RNA alone. Thermal melting studies show a marked increase in the T_m ($\Delta T_m = 26°$). Visible-region circular dichroic bands are induced when the dye is bound to RNA. These effects are also very similar to the results of studies on ethidium bromide–DNA complexes (Dalgleish, D. G., Peacoche, A. R., Fey, G., and Harvey, C. (1971), *Biopolymers 10*, 1853; LePecq, J. B., and Paoletti, C. (1967), *J. Mol. Biol. 27*, 87). Our data appear to indicate two modes of binding of the dye to RNA which are consistent with electrostatic and intercalative interactions.

Ethidium bromide is a dye which has been widely used in nucleic acid binding studies. As a drug, it has trypanocidal, antibacterial, and antiviral activities (Dickenson *et al.*, 1953; Newton, 1964). The dye inhibits DNA polymerase (Elliott, 1963) and DNA-dependent RNA polymerase (Waring, 1964). *In vitro* the dye binds to both RNA and DNA (Waring, 1965a).

Two main modes of binding to native DNA have been suggested based on the results of spectral and hydrodynamic studies. The primary and generally stronger mode of binding has been interpreted as "intercalation" where a part of the ethidium ion sandwiches between adjacent base pairs. Spectral shifts in the 480-mμ absorption band of the dye (Waring, 1965a) together with a decrease in sedimentation coefficient (LePecq and Paoletti, 1967) and an increase in viscosity (LePecq, 1965) with extent of binding occurs on formation of the complex. The hydrodynamic changes, indicative of lengthening of the DNA polymer, support the intercalation hypothesis. A decrease in buoyant density upon binding of the dye to DNA has also been observed (LePecq and Paoletti, 1967).

Hydrodynamic changes also occur in closed circular DNA in the presence of ethidium bromide. These changes can be related to changes in superhelical density due to intercalation (Crawford and Waring, 1967; Bauer and Vinograd, 1968). Recent electron microscopic studies show a 27% increase in molecular length for a linear DNA–ethidium bromide complex and a relief of superhelical twisting in closed circular DNA in the presence of ethidium bromide (Freifelder, 1971).

The second and generally weaker mode of binding is most evident at low salt and high ethidium bromide concentration. This mode is thought to be an electrostatic interaction between the phosphate groups in the double-stranded nucleic acid backbone and the dye molecules.

The same types of spectral effects have been observed when ethidium bromide binds to RNA. A number of RNAs of ill-defined secondary and tertiary structure have been studied including ribosomal (Waring, 1965a), "core" (Waring, 1965b) and tRNA (Bittman, 1969). LePecq and Paoletti (1967) postulated intercalative binding of ethidium bromide preferentially to helical regions in RNA. Waring (1965b) using spectral techniques studied binding of the dye to a group of synthetic polynucleotides. He was able to establish a relationship between the degree of secondary (helical) structure and the strength of primary binding. In these spectral studies on RNA and RNA-like polynucleotides, primary binding is considered synonymous with intercalation. However, this proposal must be viewed with some reservation since there is no supporting hydrodynamic evidence for these systems and the spectral effects in themselves are not sufficient to define a mode of binding.

In the present study the interaction of ethidium bromide with native double-stranded RNA (ds-RNA),[1] having secondary and tertiary structural characteristics and hydro-

† From the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46206. *Received July 3, 1972.*

[1] Abbreviation used is: ds-RNA, double-stranded RNA.

dynamic behavior similar to native DNA, was examined using both spectral and hydrodynamic techniques.

Materials and Methods

Purification of Penicillium chrysogenum Mycophage and Isolation of Its ds-RNA. The details of the growth conditions and purification of the mycophage will appear elsewhere (Nash *et al.*, 1973). Final purification of the mycophage was by isopycnic banding in sucrose, followed by isopycnic banding in CsCl. The banding densities were 1.20 in sucrose and 1.38 in CsCl. The ds-RNA was removed from purified mycophage by extraction with freshly distilled phenol which had been equilibrated with 0.05 M NaCl–10^{-3} M Tris–10^{-3} M EDTA (pH 7) buffer prior to use. Excess phenol was removed by dialysis against the same buffer. After removal of phenol, samples were dialyzed against 0.1–0.001 M sodium cacodylate buffer (pH 7). Cacodylate buffers were prepared by titrating a 1.0 N standard NaOH solution (Fisher Scientific Co.) with a 1.0 M cacodylic acid solution to pH 7 and dilution to a desired Na^+ concentration. Monovalent and divalent ion concentrations were monitored by atomic absorption using a Perkin-Elmer 303 atomic absorption spectrometer. When more concentrated RNA (OD_{260} >2.00) was desired, concentration of the ds-RNA was achieved by dialysis against dry sucrose. Sucrose was then removed from the ds-RNA by exhaustive dialysis against an appropriate buffer.

Ethidium bromide was obtained from Sigma Chemical Corp., Inc., and Gallard-Schlesinger Chemicals. The purity of the dye was determined using the method described by Kreishman *et al.* (1971). A small amount of impurity (~5%) was removed by recrystallization of the dye from methanol. The crystalline dye had 1 mol of methanol/mol of dye as determined by nuclear magnetic resonance (nmr) and elemental analysis.

Spectrophotometric and Spectropolarimetric Studies. The circular dichroic (CD) spectra were recorded on a Cary-60 spectropolarimeter equipped with a Model 6002 CD unit. The sample chamber was thermostatted at 26°. The measured CD is expressed as ellipticity, θ, and refers to the actual measured readings of the sample solution. The mean molar residue ellipticities, $[\theta]$, were calculated on a gram-atom of phosphorus concentration basis.

The absorption spectra were recorded on a Cary-14R spectrophotometer equipped with a thermostatted cell holder. All binding studies were performed at 25°.

Preliminary binding studies were done at constant total ethidium bromide concentration in the manner described by Waring (1965a). However, the quantitative results reported in this paper displayed in Figure 2, were obtained using a procedure quite similar to that described by Müller and Crothers (1968). In this procedure, the total RNA concentration was kept essentially constant by adding very small volumes of concentrated dye solutions to stock ds-RNA solutions in the cuvette in the spectrophotometer. The amount of dye added was calculated from the optical density at the isosbestic point (510 mμ) of the free and bound dye using $\epsilon = 4.11 \times 10^3$. The amount of bound dye was calculated using eq 1, where C_B is

$$C_B = \frac{\epsilon_F{}^\lambda C_T - A^\lambda/l}{\epsilon_F{}^\lambda - \epsilon_B{}^\lambda} \quad (1)$$

the concentration of bound dye, C_T is the total amount of ethidium bromide present, l is the cell path length, A^λ is the observed optical density at wavelength λ, and $\epsilon_F{}^\lambda$ and $\epsilon_B{}^\lambda$ are the extinction coefficients for the free and bound dye, respectively. C_B and C_F values were calculated using the optical densities at 465, 480, and 530 mμ. The C_B and C_F values calculated at these wavelengths agreed to within experimental error. The extinction coefficients for the bound form of the dye were obtained from solutions where the dye to ds-RNA phosphorus ratios were between 1–500 and 1–100. Under these conditions all of the dye present was assumed to be bound. The values of ϵ were the same in each of the buffer systems used in the present study. The extinction coefficients for free and bound dye are summarized in Table I. ds-RNA concentrations were determined using the optical density at 258 mμ and an ϵ_p of 7200. The ϵ_p value was determined by concurrent optical density determinations and phosphorus analyses on a series of ds-RNA stock solutions.

TABLE I: Extinction Coefficients of Ethidium Bromide.

mμ	ϵ_{free}	ϵ_{bound}
465	5210	2110
480	5700	2950
510	4110	4110
530	2200	3720

The binding studies displayed in Figure 2 were done at ds-RNA phosphorus concentrations ranging from 6×10^{-4} to 8×10^{-5} M in both 0.01 and 0.10 M sodium cacodylate buffer systems in a 1.0 cm cell. The concentration of ds-RNA phosphorus used in the 0.001 M sodium cacodylate buffer system was 6×10^{-5} M. The spectral data were analyzed using the Scatchard (1949) relationship

$$\frac{r}{m} = K_{ap}(B_{ap} - r) \quad (2)$$

where r is the dye bound per ds-RNA phosphorus, m is the concentration of free dye, and B_{ap} and K_{ap} are, respectively, the apparent number of binding sites and the apparent binding constant, both determined from a plot of r/m *vs* r.

Analytical Ultracentrifugation. Sedimentation studies were performed on a Beckman Model E analytical ultracentrifuge equipped with an ultraviolet scanner system. Three sample cells were run simultaneously in an AN-F rotor; one cell contained the stock nucleic acid with no dye, while each of the other cells had different dye concentrations and the same stock nucleic acid solution. The experiments were done at 20° with a rotor speed of 40,000 rpm.

Buoyant densities were determined by equilibrium banding in Cs_2SO_4. The samples each had 5.6×10^{-6} mol of RNA phosphorus/ml of Cs_2SO_4 solution and varying amounts of ethidium bromide. The studies were done in an AN-F rotor at 44,000 rpm and at 25°. Equilibrium was attained after 24 hr and data were recorded after about 26 hr. The initial density of the Cs_2SO_4 solutions were calculated from the relationship (Ludlum and Warner, 1965), $\delta^{25°} = 13.6986\eta_D^{25} - 173233$. The refractive indices (η^{25}) were measured with a Bausch and Lomb Abbe refractometer. The density at the band center was calculated by the direct method of Vinograd and Hearst (1962) using β values from Ludlum and Warner (1965).

Viscosity. Viscosities were measured using a Cannon Ubbelohde, semimicro dilution viscometer at 20°. The shear stress for this viscometer under the conditions of these ex-

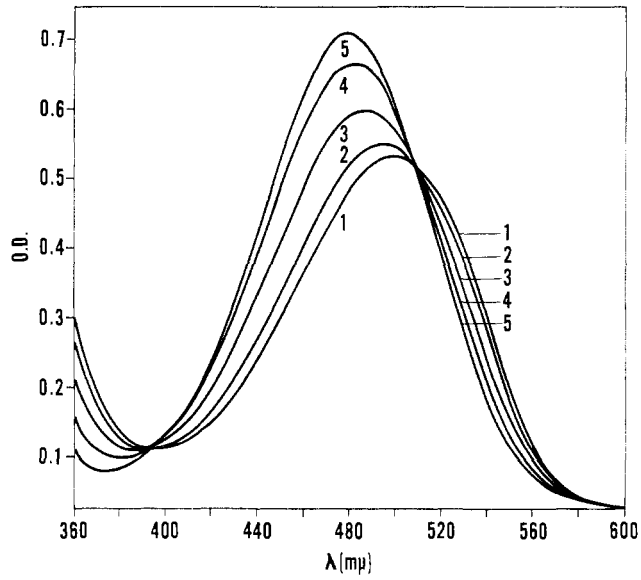

FIGURE 1: Binding of ethidium bromide to ds-RNA at constant ethidium bromide concentration. Spectra of ethidium bromide ds-RNA system at a total ethidium bromide concentration of 2.5×10^{-5} M. Double-stranded RNA concentrations are (1) 1.0×10^{-4} M, (2) 2.5×10^{-4} M, (3) 4.2×10^{-4} M, and (4) 5.2×10^{-4} M in phosphorus. Curve 5 is ethidium bromide alone. Solvent system 10^{-2} M Tris–10^{-2} M EDTA (pH 7.1); spectra taken in 5-cm cell path at 25°.

periments was estimated to be $\sim$1000 sec^{-1}. Small increments (usually 5 μl) of a concentrated ethidium bromide solution were added to a 3-ml volume of ds-RNA in the viscometer by means of a micropipet. Viscosities were measured relative to standard 0.1 M sodium cacodylate (pH 7.0) buffer. Flow times were in the range of 70–100 sec.

Thermal Denaturation. Thermal transition studies were performed in a Cary 16 spectrophotometer equipped with an electrically heated 5-position automatic sample changer block. Cuvet temperatures were monitored by a thermistor inserted in a blank cuvet containing buffer. Optical densities and temperatures for each sample were recorded continuously with a heating rate of 0.3–0.6°/min. These studies were performed in 0.001 M sodium cacodylate (pH 7.0) buffer.

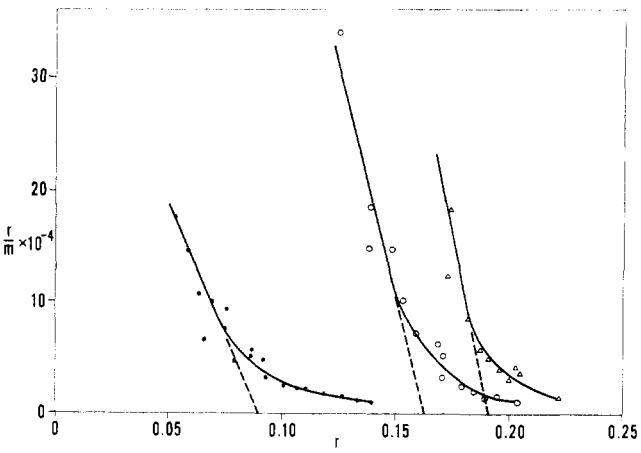

FIGURE 2: Binding isotherms at various ionic strengths. (●) 10^{-3} M sodium cacodylate (pH 7.0); (○) 10^{-2} M sodium cacodylate (pH 7.0); (Δ) 10^{-1} M sodium cacodylate (pH 7.0).

TABLE II: Binding Parameters for Ethidium Bromide–ds-RNA Complex.

Na^+	K_{ap} (M^{-1})	B_{ap}
0.001	1×10^7	0.19
0.01	8.1×10^6	0.16
0.10	4.7×10^6	0.09

Results

Characterization of the Mycophage ds-RNA. A detailed description of the physical characterization of the ds-RNA used in this study will appear elsewhere (Nash *et al.*, 1973). The material is essentially the same as that characterized by Buck *et al.* (1971) also obtained from purified mycophage. The ds-RNA from mycophage contains three species with very similar migration rates when subjected to electrophoresis on 2.5% polyacrylamide gels. This composite ds-RNA has an $s_{20,w}$ of 13.0 S and an [η] of $\sim$13.6 dl/g. Electron micrographs show a typical rodlike appearance with a calculated molecular weight of $\sim 2.0 \times 10^6$. The double-stranded character of the RNA has been firmly established by nuclease resistance (Buck *et al.*, 1971), thermal melting profile, circular dichroism, and electron microscopy (Buck *et al.*, 1971; Nash *et al.*, 1973).

Spectrophotometric Determination of Binding Equilibria. The results of the mixing of different amounts of RNA with a fixed amount of ethidium bromide are shown in Figure 1. The absorption maximum red shifts and decreases in intensity. When all of the dye present is bound, the absorption maximum of the dye shifts to about 516 mμ. An isosbestic point for free and bound dye is found at 510 mμ. These effects are similar to those seen when ethidium bromide is bound to either RNA or DNA (Bittman, 1969; LePecq and Paoletti, 1967).

Binding isotherms obtained at different Na^+ concentrations are shown in Figure 2. The curvature of the isotherms indicates at least two different modes of binding at each Na^+ concentration, and that the binding parameters are ionic strength dependent. The apparent number of binding sites and binding constants for each Na^+ concentration are summarized in Table II. The values of K_{ap} and B_{ap} were all obtained in the nearly linear region of each isotherm at low r values.

Hydrodynamic Indications of Molecular Elongation. The increase in the specific viscosity ratio Y with increasing mol of dye per mol of phosphorus (D/P) is shown in Figure 3. The quantity Y, similar to the Y parameter used in a study of actinomycin D binding to DNA by Müller and Crothers (1968), is defined as the ratio $\eta_{sp}^{\alpha}/\eta_{sp}^{0}$, where η_{sp}^{α} is the specific viscosity of the ds-RNA in the presence of dye at concentration α and η_{sp}^{0} the specific viscosity of the ds-RNA alone. As D/P increases Y increases and starts to reach a plateau at a D/P value of about 0.12. The total change in Y is about 53%.

Theoretically, the intrinsic viscosity of the complex should be determined at each ethidium bromide concentration. This necessitates an extrapolation to zero concentration of the complex, keeping the composition of the complex constant, which in practice is fairly difficult to do. Instead the approximation $\eta_{sp}/C \approx [\eta]$ was made. Figure 4 is a plot of η_{sp}/C *vs.* C for the ds-RNA alone in 0.1 M cacodylate buffer (pH 7), with estimated error limits. The extrapolated value of [η] is

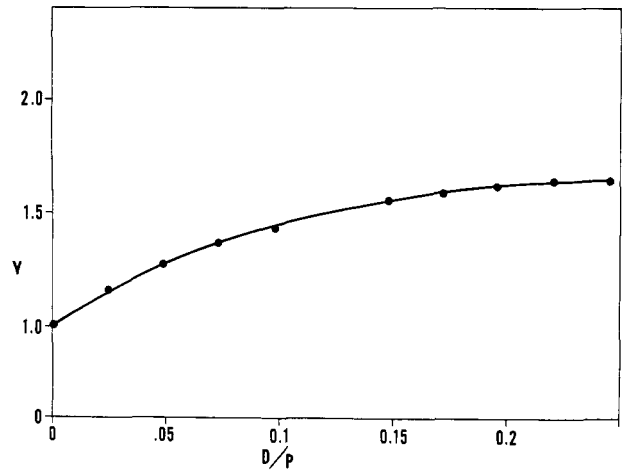

FIGURE 3: Viscosity ratio, Y, increase with added ethidium bromide. The ratio of specific viscosities (Y) of the complex mixture and the ds-RNA alone *vs.* added mol of ethidium bromide per mol of phosphorus (D/P). ds-RNA concentration 1.71×10^{-3} M phosphorus. Buffer 0.1 M sodium cacodylate (pH 7.0).

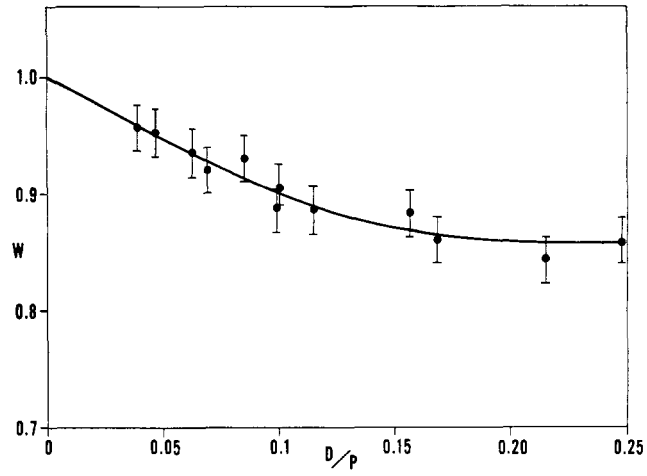

FIGURE 5: Sedimentation coefficient ratio, W, decrease with added ethidium bromide. The ratio of sedimentation coefficients, W, of the complex and the ds-RNA alone *vs.* D/P. Temperature 20°.

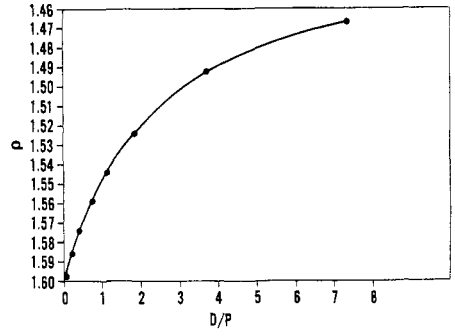

FIGURE 6: Buoyant density difference of ds-RNA ethidium bromide complex in Cs_2SO_4. ρ, the measured buoyant density in Cs_2SO_4, is plotted as a function of (D/P), dye to RNA phosphorus ratio.

probably somewhat low because of shear effects in the viscometer.

Sedimentation and Buoyant Density. Since the specific viscosity ratio Y increases upon binding of the dye a corresponding decrease in the sedimentation coefficient ratio W would be expected if the ds-RNA molecule is undergoing an elongation in the complex (Müller and Crothers, 1968). W is defined as the ratio of the sedimentation coefficient of the dye ds-RNA complex to the sedimentation coefficient of the ds-RNA alone. The decrease in W as D/P increases is shown in Figure 5. A plateau value of W is reached at about the same D/P value as Y in the viscosity study.

With DNA a decrease in buoyant density of the dye–DNA complex as compared to free DNA has been observed (LePecq and Paoletti, 1967). An analogous decrease is seen (Figure 6) in the buoyant density of the dye ds-RNA complex in Cs_2SO_4 as the value of D/P increases.

Effects of Thermal Melting. The addition of ethidium bromide to ds-RNA has three effects on the thermal denaturation profile of the nucleic acid. As shown in Figure 7, addition of the dye increases the temperature of the midpoint of the transition, changes the transition breadth, and results in a pronounced multiphasic transition profile. The changes in the transition midpoint, ΔT_m, and the changes in the transition breadth, ΔT_{30-70} are summarized in Table III. As ethidium bromide is added the midpoint of the transition increases to a value of ΔT_m of 26.2° at a D/P of 0.370.

Circular Dichroic Effects. Ethidium bromide exhibits no intrinsic optical activity. However, when the dye is bound to DNA, induced circular dichroic bands appear in the visible spectral region (Dalgliesh *et al.*, 1971). The same type of induced CD spectrum is found when ethidium bromide is bound to ds-RNA as seen in Figure 8. Circular dichroic bands at

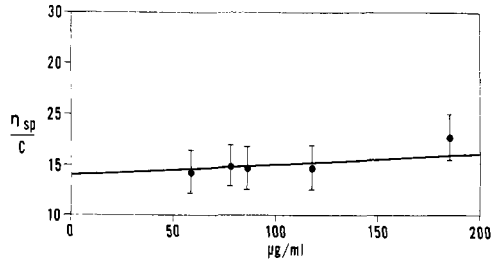

FIGURE 4: Intrinsic viscosity of *P. chrysogenum* ds-RNA. A plot of η_{sp}/C (dl/g) *vs.* C (μg/ml) for *P. chrysogenum* ds-RNA. The point at 180 μg/ml was weighed less heavily than those at lower concentrations in determining the line. Extrapolation to zero concentration gives $[\eta] = 13.6$ (dl/g). Buffer 0.1 M sodium cacodylate (pH 7.0). Temperature 20°.

TABLE III: Thermal Denaturation of ds-RNA in the Presence of Ethidium Bromide.[a]

D/P	ΔT_m (deg)	ΔT_{30-70} (deg)
0	0	2.8
0.037	3.9	7.8
0.185	19.6	5.5
0.370	26.2	3.1

[a] ΔT_m = temperature of midpoint of thermal transition with added ethidium bromide relative to temperature of midpoint of transition of RNA alone. ΔT_{30-70} = breadth of thermal transition in °C from 30% hyperchromicity to 70% hyperchromicity. Buffer system: 0.001 M sodium cacodylate (pH 7.0).

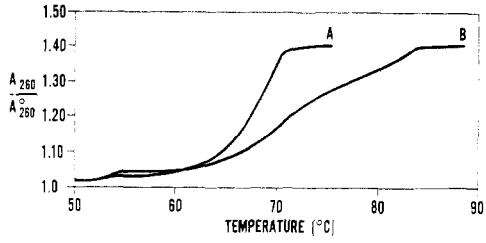

FIGURE 7: Effect on thermal denaturation profile of added ethidium bromide. Curve A is the thermal transition profile of ds-RNA alone. Curve B was obtained under the same ionic strength conditions with the addition of 0.037 mol of ethidium bromide/mol of RNA phosphorus.

510, 375, 330 and 315 mμ are easily detected. The CD spectrum of the ds-RNA–dye complex also exhibits some marked changes in the region below 310 mμ as shown in Figure 9. The interpretations of these spectra are complicated by the fact that the ds-RNA molecule itself has a strong CD spectrum in this region. The spectrum of the ds-RNA without dye is quite similar to that reported by Cox *et al.* (1971) for mycophage RNA as well as those of other ds-RNAs such as Rice dwarf virus RNA (RDV RNA) (Samejima *et al.*, 1968). The band maximum of our material is at 261 mμ and has a $[\theta]$ of 2.9×10^4. This is significantly larger than the value of 2.4×10^4 reported for RDV RNA. The $[\theta]$ of -2.9×10^3 for the 295-mμ trough is essentially the same as that reported for RDV RNA.

Discussion

Three electrophoretically distinct species are present in *P. chrysogenum* ds-RNA. These species could represent slightly different molecular weights and/or conformational or compositional differences. However, the existence of these three species is not readily discernible by other techniques including gradient ultracentrifugation and electron microscopy—which tend to indicate the existence of one homogeneous species (Nash *et al.*, 1973). Electron micrographs along with the values of $s_{20,w} = 13.0$ S and $[\eta] = 13.6$ describe a rodlike double helix hydrodynamically similar to native DNA (Eigner and Doty, 1965).

Actually, the *P. chrysogenum* ds-RNA is better characterized and more homogeneous than much of the DNA (calf thymus) and RNA used in previous binding studies. The

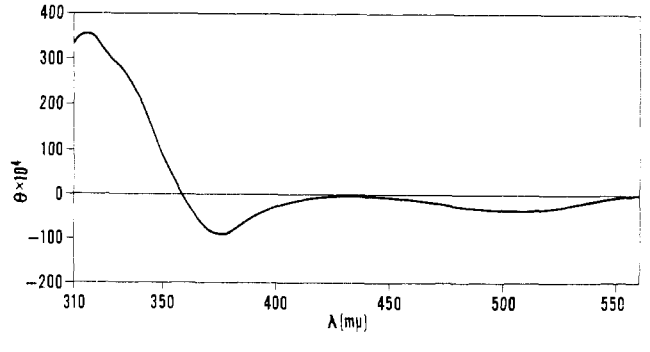

FIGURE 8: Visible-region-induced CD spectrum of ethidium bromide bound to ds-RNA. θ is the observed ellipticity for a solution with ethidium bromide concentration of 2.68×10^{-5} M. The concentration of ds-RNA is 6.32×10^{-4} M in phosphorus. Spectrum taken at 26° in a 5-cm path-length cell; buffer 0.10 M sodium cacodylate (pH 7.0).

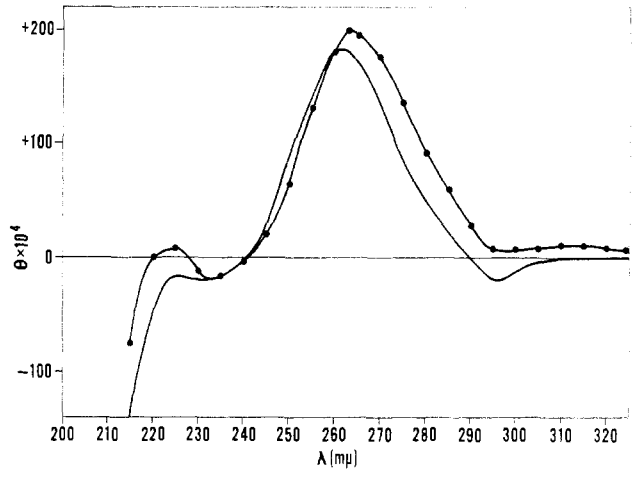

FIGURE 9: Ultraviolet CD spectrum of ds-RNA in the absence and presence of ethidium bromide. θ is the observed ellipticity. The RNA concentration is 6.32×10^{-4} M in phosphorus. Spectra are shown in the absence (——) and the presence (●) of 2.68×10^{-5} M ethidium bromide, path length 1.0 mm; 26° buffer, 0.10 M sodium cacodylate (pH 7.0).

slight degree of heterogeneity of the ds-RNA is probably of no importance in our binding studies except possibly in the case of the thermal melting experiments. The addition of ethidium bromide to ds-RNA solutions results in a thermal transition with a pronounced multiphasic profile. This effect could be due to differential binding to each of the three ds-RNA species; however, preferential binding of the dye to particular regions in the same molecule would also lead to the observed multiphasic transition profile. The isolation of the different RNAs would resolve the source of these thermal profile effects.

The shape of the Scatchard isotherms shown in Figure 2, as well as the ionic strength dependency of the binding is, quite similar to those found for native DNA binding of the dye. Waring (1965a) studied T2 DNA and obtained a value for K_{ap} of 2×10^6 in a 0.04 M Tris-HCl buffer. Although our studies have not been performed under identical experimental conditions, the range in the K_{ap} from 4.7×10^6 to 10×10^6 is of comparable magnitude. The same kind of curvature is observed in our Scatchard plots as reported by Waring for T2 DNA. This curvature is indicative of at least two different modes of binding. One of these modes is strongly ionic strength dependent and probably is an electrostatic interaction of the ethidium ions with the phosphate groups of the nucleic acid backbone. Thus, the ionic strength dependence of the binding is primarily the result of competition for the available electrostatic binding sites. At low ionic strength (0.001 M Na^+) one drug molecule is bound per five phosphate groups, while at higher ionic strength (0.10 M Na^+), about one dye molecule is bound per ten phosphates.

Intercalation represents another (primary) mode of binding of ethidium bromide to DNA and results in a molecular elongation. These phenomena in turn are reflected in changes in the hydrodynamic properties of the DNA molecule. Our results with ds-RNA demonstrate the same hydrodynamic effects: a 53% increase in viscosity and a 15% decrease in sedimentation coefficient. Thus, the obvious conclusion is that the primary mode of binding of ethidium bromide to ds-RNA is probably intercalation.

The similarity in the effect of ethidium bromide binding on the physical properties of ds-RNA and native DNA is also re-

flected in the T_m and CD results. Substances which interact with nucleic acids change both the nucleic acids' melting profile and the position of its midpoint. The T_m of calf thymus DNA is increased some 30° in the presence of ethidium bromide (LePecq and Paoletti, 1967), while the melting temperature of our ds-RNA increases by 26.2° upon the addition of 0.370 mol of dye/mol of RNA phosphorus. The increase in transition breadth of the ds-RNA melting profile at low values of D/P is probably a result of interaction at primary binding sites. The subsequent decrease in transition breadth at high values of D/P may reflect electrostatic binding of the dye which would tend to decrease interphosphate repulsion energy, stabilizing the structure and increasing the cooperative nature of the transition.

The results of the circular dichroic studies again demonstrate the similarity in the interaction of ethidium bromide with ds-RNA and native DNA. As has been reported previously for native DNA (Dalgleish *et al.*, 1971), the binding of the dye to ds-RNA results in the generation of circular dichroic bands. Though their shapes and positions are similar, the exact location of these bands does not appear to be the same for ds-RNA as for native DNA. For example, the ds-RNA–ethidium complex exhibits a band at 375 mμ, while the DNA–ethidium complex exhibits a similar band at 380 mμ. However, the conditions under which the two sets of measurements were made were quite different. Therefore, the CD differences cannot, at this time, be related to differences in the mode of interaction of the dye with the two types of nucleic acids.

The ultraviolet region CD spectrum of the ds-RNA–dye complex is difficult to interpret because of potential contributions by both the dye and the nucleic acid. However, much of the difference between the spectrum of the complex and that of ds-RNA, especially in the 240- to 285-mμ region, can reasonably be assumed to be due to the ds-RNA rather than the dye. The ethidium bromide has an appreciable absorption band in this region only at the longer wavelength. The 285-mμ band is red shifted upon interaction with the nucleic acid. The overall CD changes in the 240- to 280-mμ region observed upon formation of the complex could therefore be related to a conformational transition that occurs in the ds-RNA upon binding of the drug. Further studies on these CD effects are presently being performed in this laboratory.

A direct comparison of our results with those obtained from studies on other RNAs is not as clear as the comparison with native DNA. Binding isotherms observed in these systems range from one with constant curvature obtained from rRNA (Waring, 1965a) comparable to denatured DNA, to an isotherm obtained from tRNA (Bittman, 1969) comparable to our results with ds-RNA.

A small indication of binding was observed by Waring (1965b) to "core RNA." However, with the ds-RNA analog poly(I)·poly (C), he obtained binding parameters of B_{ap} = 0.20 and $K_{ap} = 6.0 \times 10^6$ in 0.04 M Tris-HCl buffer (pH 7.9) which are in the general range of the values of these parameters determined by us for ds-RNA (Table II).

We have shown that ethidium bromide interacts strongly with ds-RNA. The evidence for this is very similar to that used to interpret DNA binding as intercalation. The view that the drug intercalates preferentially into double-stranded regions of other RNAs (LePecq and Paoletti, 1969) is therefore a tenable if unproven hypothesis. However, the interaction may not be exclusively with double-stranded regions since Kreishman *et al.* (1971) have recently shown intercalative interaction in single-stranded ribopolymers.

ds-RNA exists in an A-like conformation in solution (Arnott, 1971) where the base pairs are tilted relative to the helix axis. While we believe our results support the concept of intercalative interaction of ethidium ion with ds-RNA, some other nonelectrostatic external binding mode affecting this geometry may occur. An A → B transition for DNA is accompanied by a 32% increase in rise per residue (Arnott, 1971). If the binding of the dye should cause the molecular geometry of the ds-RNA to become more B like than A like, some or all of the molecular length changes could be accounted for on this basis. A more certain elucidation of the ds-RNA–dye interaction therefore awaits further studies such as X-ray diffraction and the formulation of appropriate molecular models.

In conclusion, strong similarities exist among the measured physical properties of DNA and ds-RNA complexes with ethidium ion in solution. The binding isotherms and the changes in hydrodynamic properties are indicative of two modes of dye–nucleic acid interaction. The ionic strength dependent weaker mode probably represents electrostatic interaction between the quaternary ammonium cation of the dye and the ionized phosphate groups of the nucleic acid backbone. The stronger mode leading to characteristic hydrodynamic changes can be interpreted as intercalation. The parallelism observed in the effects of dye binding on the properties of both DNA and ds-RNA is both striking and strong suggestive of intercalation. We feel, however, that this mode of binding to ds-RNA has not yet been unambiguously established.

Acknowledgment

The authors gratefully acknowledge helpful discussions with Professor Struther Arnott of Purdue University and the generous help of Dr. L. F. Ellis in providing the electron micrographs of ds-RNA. Appreciation is also extended to Mr. Max M. Marsh for his helpful discussions and comments on this manuscript.

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Reconstitution of 50S Ribosomal Subunits from Protein-Free Ribonucleic Acid†

S. Fahnestock,‡ V. Erdmann,§ and M. Nomura*

ABSTRACT: Previous reconstitution studies on the 50S ribosomal subunit of *Bacillus stearothermophilus* used as starting material a complex of 23S rRNA with a 50S ribosomal protein, L3. This protein can be removed from 23S RNA at pH 2.0 in the presence of 4 M urea and 0.5 M Mg^{2+}, and active 50S subunits can then be reconstituted from the resulting protein-free 23S RNA. Thus, the 50S ribosomal subunit can be completely self-assembling. Protein L3 is not important for the physical assembly of the 50S subunit, but is required for the reconstitution of active particles. Particles reconstituted without L3 are deficient in several different ribosomal functions.

The large ribosomal subunit from *Bacillus stearothermophilus* can be reconstituted *in vitro* from dissociated RNA and protein fractions (Nomura and Erdmann, 1970). In previous studies we have made use of a reconstitution system utilizing RNA and protein fractions obtained by dissociation with 4 M urea–2 M LiCl (Nomura and Erdmann, 1970; Erdmann *et al.*, 1971a; Fahnestock and Nomura, 1972). As we have previously described, 23S rRNA prepared in this way retains several proteins still bound to it (Nomura and Erdmann, 1970; Erdmann *et al.*, 1971b). One of these proteins, which we have designated L3, is present only in small amounts in the urea–LiCl protein fraction, being found mostly in the RNA fraction; several other proteins which are found in the RNA fraction are present in larger amounts in the protein fraction.

Since RNA obtained by the urea–LiCl precipitation method ("urea–LiCl RNA" in this paper) retains bound protein, the above system cannot strictly be considered a complete reconstitution. It is possible that the 23S RNA–L3 complex retains some structural features of the 50S subunit which might not be retained by the free RNA and protein. In order to determine whether the 50S ribosomal subunit can be entirely self-assembling *in vitro* or whether perhaps the L3–RNA complex retains some important assembly information which might result from some extraribosomal influence *in vivo*, it is necessary to reconstitute the 50S subunit from protein-free RNA. Here we describe the total reconstitution of the *B. stearothermophilus* 50S subunit, together with some properties of the protein L3.

Materials and Methods

Ribosomes were obtained from *B. stearothermophilus* and washed through sucrose and 0.5 M NH_4Cl as described previously (Fahnestock and Nomura, 1972). Purified 50S subunits were obtained as described (Erdmann *et al.*, 1971a).

A mixture of (undissociated) 50S and 30S ribosomal subunits was treated with 4 M urea–2 M LiCl for 36–48 hr at 0° to obtain RNA and protein fractions (Nomura and Erdmann, 1970). The RNA fraction was freed of remaining protein by treatment with 4 M urea–0.5 M $MgAc_2$ (pH 2.0). The RNA pellet obtained from the urea–LiCl treatment, after washing with 4 M urea–2 M LiCl, was redissolved in 6 M urea to a concentration of 10–20 mg/ml. To this solution was added one-third volume 2 M $MgAc_2$ which had been adjusted to pH 2.0 with HCl. After 1 hr at 0°, the precipitated RNA was collected by centrifugation, suspended in a buffer (TMA-II) containing 10 mM Tris-HCl (pH 7.4), 0.3 mM $MgCl_2$, 30 mM NH_4Cl, and 6 mM 2-mercaptoethanol, and dialyzed until dissolved (4 hr) against the same buffer. The supernatant containing protein L3 (crude L3) was dialyzed 6 hr against a buffer (TRI) composed of 30 mM Tris-HCl (pH 7.4), 20 mM $MgCl_2$, 1 M KCl, and 6 mM 2-mercaptoethanol.

Purification of L3. Protein L3, identified by its mobility on two-dimensional polyacrylamide gel electrophoresis (see Figure 5), was purified from the pH 2 urea–Mg supernatant by chromatography on carboxymethylcellulose. Protein from 100 mg of 70S ribosomes was applied to a 3-ml column of Whatman CM52 equilibrated with buffer A, composed of 16 ml of pyridine, 9.6 ml of formic acid (88%), and 1 ml of 2-

† From the Institute for Enzyme Research and Departments of Biochemistry and Genetics, University of Wisconsin, Madison, Wisconsin 53706. *Received August 31, 1972.* Paper No. 1579 of the Laboratory of Genetics. This work was supported in part by the College of Agriculture and Life Sciences, University of Wisconsin, and by grants from the National Institute of General Medical Sciences (GM-15422) and the National Science Foundation (GB-31086X). S. F. was supported by a Postdoctoral Fellowship from the American Cancer Society and V. E. by a Postdoctoral Fellowship from the National Institutes of Health.

‡ Present address: Department of Biochemistry, Pennsylvania State University, University Park, Pa.

§ Present address: Max-Planck-Institut für Molekulare Genetik, 1 Berlin (Dahlem), Germany.